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(54) Title: DNA-ANALYSIS METHOD INVOLVING GENE AMPLIFICATION AND MAGNETIC PARTICLES

(57) Abstract

A method for gene assaying in a test medium comprising denaturing DNA in the test medium, contacting the test medium under hybridizing conditions with heat resistant DNA polymerase, deoxynucleotides, two different oligonucleotide primers, one of which is soluble, whereas the other is bonded to superparamagnetic particles, amplifying DNA, removal of the DNA strand which is not bonded to the particles, as well as free oligonucleotide primers, and detecting amplified material.

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DNA-ANALYSIS METHOD INVOLVING GENE AMPLIFICATION AND MAGNETIC PARTICLES

The present invention is a new method of gene assaying in humans, animals, plants, and microorganisms. Such assays previously required complicated procedures and time consuming techniques. What is new in this invention is that super-paramagnetic particles, e.g. Ugelstad spheres, are used to isolate genes, and that these isolated genes, while attached to the particles, are copied 10^5-10^6 times and assayed. Since isolation, copying, and assay occur on the surface of a solid superparamagnetic phase, gene assays are considerably more simple, rapid, less labour consuming, and more sensitive. Also, this process may readily be automated.

Solid phases which are commonly used for immobilizing nucleic acids comprise microporous membranes of nitrocellulose or nylon. Hybridization is carried out by immersion of the membrane in a hybridization liquid which contains the complementary nucleic acid sequence. This technique entails a long hybridization period as well as a low degree of hybridization.

Identification of genes may be carried out by probing with complementary DNA sequences (DNA-probes). In restriction fragment length polymorphism assays (RFLP)-assays total DNA is treated in the cells by a restriction endonuclease. Then the fragments are separated electrophoretically and are blotted on filters before probing with a DNA sequence which is complementary to the DNA region of interest. Restriction endonuclease cuts the DNA at palindrome sequences (specific seats of restriction endonuclease) which is in bonding imbalance with allele sequences. Consequently, the size of DNA fragments and, thus, the band patterns may vary, dependent on the allele variations within the region reacting with the utilized DNA-probes. The RFLP technique is a time consuming and complicated method, requiring several days of work for completion. Also, bands which are detected in RFLP-

blot need not show information of importance, as several restriction seats are common to several alleles. This makes it difficult to evaluate RFLP-blots.

In the polymerase chain reaction technique (PCR) the region of the genome containing the gene of interest is amplified before probing with oligonucleotides which are complementary to constant or to all polymorphic sequences within the amplified DNA-region (cf. 1.3). This technique substantially reduced the time required for carrying out gene assays. But even this method requires extensive handling of the test material, including an amplification step in a test tube, upon which the amplified DNA-material is transferred to a nitrocellulose membrane and analysed with a gene probe.

The object of the present invention is to provide a new method for gene assay in humans, animals, plants, and microorganisms, where the steps of the assay occur on the surface of a solid superparamagnetic phase, thus, resulting in a gene assay that is more simple and rapid, less labour consuming, and more sensitive.

According to the present invention a method for gene assay in a test medium is thus provided, and said method is characterized by

a) denaturing DNA in the test medium,
b) contacting the test medium under hybridizing conditions with heat-resistant DNA-polymerase, deoxynucleotides, two different oligonucleotide primers, one of which is soluble, whereas the other is bonded to superparamagnetic particles,
c) amplification of DNA,
d) removal of the DNA strand which is not connected with the particles, as well as free oligonucleide primers,
e) detecting amplified material.

The test medium may be placed in test wells or the wells of a transparent multi-well plate. The multi-well plate is then covered by a lid and is placed in a metal block which is brought into close contact with the lid, bottom, and sides of each well on the plate. The temperature in this metal block may be rapidly and automatically changed. To destroy the cells and denature the DNA, the temperature of the metal block is adjusted to 100°C, and the multi-well plate is incubated at said temperature for 5-10 min. A temperature which depends on the length and the purine/pyrimidine ratio of the oligonucleotide primer is set, and heat resistant DNA polymerase, deoxynucleotides, two different oligonucleotide primers (one soluble and one bonded to superparamagnetic particles) are added to each well. Amplification of DNA is carried out by alternating the temperature of the metal block between a temperature determined by the primer used, e.g. approx. 20-85°C, and 90°C during a number of cycles depending on the degree of amplification required for the test medium to be assayed. The incubation period is 1-2 min. for each cycle at a temperature determined by the used primer, and 0.5 min. at 90°C. The multi-well plate is then placed on the magnet and the walls are washed at 90°C to remove the DNA strand which is not bonded to particles, as well as the free oligonucleotide primers. Amplified material may then be detected either by (A) specific probing with oligonucleotides and demonstration of labelled nucleotides without, or after renewed polymerase reaction, or (B) specific probing, polymerase reaction, and demonstration of double-strand DNA with labelled intercalating substances, or (C) assayed by DNA sequence assay.

A.

Specific probing with oligonucleotides may be carried out in three different manners:

A1.

Probing with labelled oligonucleotide probes may be carried out by adding a biotinylated oligonucleotide probe to each well at a temperature determined by the probe used. The multi-well plate is then incubated at said temperature for 5-10 min. Then the multi-well plate is placed on a magnet and each well is washed. To biotine labelled probes which are bonded to amplified DNA avidine (or streptavidine) connected to enzymes, fluorochromes, etc. is added. Bonding of biotinylated probes is then analyzed by use of relevant detecting systems for the different labels, as chemiluminiscense for detection of peroxidase-labelled avidine. In stead of biotine-labelled probes, radioactive labelled probes may be used.

A2.

Production of labelled DNA by the aid of specific oligonucleotide primers may be carried out by adding a mixture of non-labelled and biotinylated deoxynucleotides, DNA polymerase, and an oligonucleotide probe to particle-bonded amplified DNA, and incubating at a temperature determined by the length and composition of this probe. Particle-bonded amplified single-strand DNA will serve as a template, whereas the oligonucleotide probe will serve as a primer. In this manner, a DNA strand containing labelled nucleotides will be produced. Remaining steps of the probing method are carried out as described under A1. In stead of biotine-labelled nucleotides, radioactive-labelled probes may be used in the same manner as in conventional techniques.

B.

The production of DNA by use of a specific oligonucleotide probe and following detection of double-strand DNA by use of intercalating substances may be carried out by adding a mixture of deoxynucleotides, DNA-polymerase, and an oligonucleotide probe to particle-bonded amplified single-strand DNA, and incubating at a temperature determined by the length and nucleotide composition of the probe. Particle bonded

single-strand DNA will serve as a template, whereas the oligonucleotide probe may serve as a primer and synthesis of the DNA-strand complementary to the template will occur. This double-strand DNA may now be demonstrated by adding a biotinylated compound which intercalates non-specifically with double-strand DNA.

The remaining steps of detection are carried out as mentioned under A1.

C. Sequence analysis

The superparamagnetic spheres with bonded amplified DNA are heated to 95°C for 5 minutes and washed, and DNA-polymerase, and soluble primer are added once more (this time, e.g. labelled 32P), and the dideoxy method (ref. 2) may be used for sequencing. Before analysis the sample is heated for some minutes, e.g. for 2 min. to 95°C, and DNA bonded to superparamagnetic spheres is removed by the aid of a magnet. The sequence analysis per se, e.g. by polyacrylamid gel electrophoresis, is in this case carried out on the soluble fraction.

The fundamentally new feature of the present invention is that superparamagnetic particles bonded to oligo-nucleotides are at the same time used to (1) isolate (catch) relevant DNA sequences from various biological matter (eukaryotes) or microorganisms, (2) for a solid phase on which DNA is amplified by the polymerase chain reaction, and (3) for a solid carrier on which detection of amplified material is carried out by specific probing with use of labelled allele or locus-specific oligonucleotides which may also function as primers for a polymerase reaction for further incorporation of labelled nucleotides and, consequently, enhanced sensitivity, or in demonstration of double-strand DNA with intercalating substances. For the above mentioned methods of detection the specificity of the gene assay may be mediated both by the oligonucleotide primers on the particles, since

amplification of DNA is only achieved after base pairing of oligonucleotide primers to complementary sequences, and in the probing step.

The advantages of the present invention are many. Bonding of the amplified DNA material to superparamagnetic particles permits both probing and analysis to be carried out in the same container which was used for amplification and isolation of the DNA. This will reduce handling and transfer of the test medium between different containers during production and permit simple and automatic production of the test medium. Since the amplified DNA is bonded to the surface of superparamagnetic particles, washing in the steps of catching, probing, and detection can be carried out readily, efficiently, and very quickly by use of a magnet, which permits complete automation of the entire asssay.

Probed amplified material on the particles may be focused in a small spot on the bottom of the U-shaped micro-wellplate in less than a second by utilizing a magnet. This will result in much improved sensitivity of the assay system, since the intensity of liberated energy per mm^2 will increase when the surface of the spot is reduced.

A DNA strand is amplified on the surface of magnetic particles, and this strand which may readily be separated from the non-bonded strand after PCR-amplification may then be used for a template for synthesis of a biotinylated DNA strand initiated after specific annealing of an allele or locus specific oligonucleotide probe complementary to the particle bonded DNA strand. The degree of labelling and the sensitivity of the analysis are, thus, greatly enhanced. By using particles bonded to oligonucleotides, only relevant DNA is amplified and isolated. This is specially advantageous when the technique is used to identify infectious DNA (like virus and bacteria) in biological liquids or tissue from eukaryotic organisms.

It is, furthermore underlined that the above disclosed method for isolating single-strand DNA may also be used for sequence analysis.

DNA sequence analysis requires a series of operational and time consuming steps before the sequence analysis proper. They comprise introduction of a DNA sequence of interest, e.g. in plasmids, infection by bacteria of the latter, cloning of bacteria, expansion of bacteria clones, purification of plasmid from the same, "cutting out" the DNA sequence of interest by restriction enzyms, purification of this DNA sequence which may then be sequence analysed. By amplification using superparamagnetic spheres the labour consuming steps carried out before sequence analysis proper can be omitted.

Ref. 1: R.K. Saiki, T.L. Bugavan, G.T. Horn, K.B. Mullis and H.H. Erlich. Nature 324, 163.166, 1986.

Ref. 2: F. Sanger, S. Miklen and A.R. Coulson. Proc. Nat. Acad. Sci. USA. 74, 5463-5467, 1977.

Ref. 3: R.K. Saiki, D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis & H.A. Erlich. Nature 239, 487-491, 1988.

PATENT CLAIMS:

1.
A method for examination of genes in a test medium, c h a r a c t e r i z e d i n that it comprises:
a) denaturing DNA in the test medium,
b) contacting the test medium in hybridizing conditions with heat resistant DNA-polymerase, deoxynucleotides, two different oligonucleotide primers, one of which is soluble, whereas the other is bonded to super-paramagnetic particles,
c) amplification of DNA,
d) removal of the DNA strand which is not bonded to the particles, as well as free oligonucleotide primers,
e) detecting amplified material.

2.
A method as stated in claim 1, c h a r a c t e r i z e d i n that the DNA is denatured at a temperature in the region of 95-105°C, preferably 100°C, and for a period of 5-10 minutes.

3.
A method as stated in claim 1, c h a r a c t e r i z e d i n that the denatured test medium is contacted with heat resistant DNA polymerase, deoxynucleotides, two different oligonucleotide primers, one of which is soluble, whereas the other is bonded to superparamagnetic particles, where the amplification temperature is determined by the length and nucleotide composition of the primers.

4.
A method as stated in claim 1, c h a r a c t e r i z e d i n that the DNA is amplified in a number of cycles by alternating the temperature during a period of 1-2 minutes at amplification temperature and for 0.5 min. at 85-95°C, preferably 90°C.

5.
A method as stated in claim 1, characterized in that after heating for approx. 2 minutes to 95°C, the DNA strand which is not bonded to the particles, as well as free oligonucleotide primers are removed by washing and use of a magnet.

6.
A method as stated in claim 1, characterized in that the amplified material is detected with a specific labelled probe, if desired by specific probing and another polymerase reaction with marked nucleotides, if desired with labelled intercalating substances which are bonded to this double-strand DNA.

7.
A method as stated in claim 1, characterized in that upon amplification of DNA removal and detection under items (d) and (e) are carried out by addition of a soluble primer which may, if desired, be labelled, a mixture of dideoxynucleotides and deoxynucleotides, one of which may be labelled, a DNA-polymerase; the reaction is permitted to go on for some minutes, heating is carried out for approx. 2 minutes at 95°C, the superparamagnetic particles with their DNA are removed, and DNA sequence analysis of the soluble material is carried out.

8.
A method as stated in claims 1, 2, and 6, characterized in that after denaturing DNA in the test medium the test medium is contacted under hybridizing conditions with heat resistant DNA polymerase, deoxynuclotides, two different oligonucleotide primers, and the DNA is amplified, upon which superparamagnetic spheres are added with a specific probe and the polymerase reaction is carried out at least once, followed by washing (by use of magnet), denaturing, and adding labelled deoxynucleotides, soluble

primer, heat resistant DNA polymerase, amplifying, denaturing, washing (bu use of magnet) and detecting labelled single-strand DNA on the spheres.

INTERNATIONAL SEARCH REPORT

International Application No PCT/N089/00050

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC 4	C 12 Q
	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A2, 0 265 244 (AMOCO CORPORATION) 27 April 1988 see particularly the figures, the claims and example 1 & JP, 63188399 ZA, 8707772	1-8
Y	US, A, 4 672 040 (LEE JOSEPHSON) 9 June 1987 see in particular the abstract, col. 18 line 29 to col. 20, the claims & EP, 0125995 JP, 60001564 US, 4554088 US, 4628037 US, 4695392 US, 4695393 US, 4698302	1-8 -

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
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Swedish Patent Office	Mikael Gunson Bergstrand

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	WO, A1, 86/05815 (GENETICS INTERNATIONAL INC.) 9 October 1986 see particularly the abstract and the figures & EP, 0216844	1-8
Y	EP, A2, 0 229 701 (CETUS CORPORATION) 22 July 1987 see in particular the abstract, line 9, the examples & JP, 62217161	1-8
Y	EP, A2, 0 204 510 (AMOCO CORPORATION) 10 December 1986 see the figures & JP, 61293399	1-8
Y	Kjemi, July 1987, (J. Afseth et al.) pages 63-64, "Nye perspektiver i bioseparasjon", see page 64, col. 3, lines 7-10	1-8